Plant Cell Rep (2002) 21:359–364
DOI 10.1007/s00299-002-0526-0

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Agrobacterium tumefaciens-mediated transformation of *Ocimum basilicum* and *O. citriodorum*

Received: 21 March 2002 / Revised: 14 August 2002 / Accepted: 15 August 2002 / Published online: 19 October 2002

Abstract Transgenic basil (*Ocimum* spp.) was produced for the first time using an *Agrobacterium tumefaciens*-mediated transformation system. β-Glucuronidase (GUS) transient expression experiments showed a 96% or higher infection rate using *Agrobacterium* strains GV3101 and EHA105. Inoculated leaf explants were regenerated on Murashige and Skoog medium supplemented with thidiazuron, naphthaleneacetic acid and kanamycin. Two sweet basil (cvs SBA28 and Purple Ruffles, *O. basilicum)* and two lemon basil (cv Sweet Dani, *O. citriodorum)* plants were regenerated after in vitro cultivation. Stable transformation of both species was verified by polymerase chain reaction amplification (PCR) of both the *gus* (697 bp) and *nptII* (320 bp) gene fragments and by Southern blot analysis for the *gus* gene. Inheritance of the transgenes was verified by the GUS colorimetric assay and PCR analysis of the T1 generation of *Ocimum* transgenic plants, and the observed segregation ratio (3:1) indicated a single gene for GUS. Transgenic T1 plants exhibited identical phenotypic characteristics and accumulated high concentrations of linalool (118.98 μg g^{-1}), 1.8-cineole (20.86 μg g^{-1}), eugenol (69.3 μg g^{-1}), neral (468.61 μg g^{-1}) and geraniol (649.1 μg g^{-1}) in volatile oil profiles similar to those of the non-transgenic controls.

Keywords *Ocimum* spp. · Transformation · Gus expression · Essential oils · *Agrobacterium*

Communicated by G.C. Phillips

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Abbreviations *GUS*: β-Glucuronidase · *Kan*: Kanamycin · *Kan^r*: Kanamycin resistant · *NAA*: α-Naphthaleneacetic acid · *NPTII*: Neomycin phosphotransferase II · *PCR*: Polymerase chain reaction · *TDZ*: Thidiazuron

Introduction

The genus *Ocimum* (Lamiaceae), with 30 species, naturally occurs in tropical and subtropical regions and is considered to be an important culinary herb and source of aromatic essential oils (Paton and Putievsky 1996). Sweet basil, *O. basilicum* L., is the major culinary herb and essential oil source of this genus (Lawrence 1992). Volatile essential oils from *Ocimum* include many terpenes (e.g. linalool, citral, 1.8-cineole) and phenylpropanoids (e.g. methyl chavicol, eugenol) produced in specialized glandular trichomes (Charles et al. 1990; Gang et al. 2001). Essential oils of basil accumulate and are stored in the peltate glands where specifically transcribed genes are active (Gang et al. 2001). This compartmentalization makes basil an ideal model system for the genetic modification of essential oil biosynthesis by transformation with new genes or the alteration of existing gene activity. There are many chemotypes of *Ocimum* spp., including ones rich in linalool, methyl chavicol, citral, eugenol, 1.8-cineole or methyl-*E*-cinnamate (Charles et al. 1990). Our laboratory has used traditional breeding to develop new cultivars of basil exhibiting traits such as unique aromas, distinct growth characteristics and a high tolerance against fusarium wilt (*Fusarium oxysporum* f. sp. *basilicum)*, a worldwide disease that plays a major limiting role in basil production. The development of a transformation system for basil could allow the introduction of novel qualitative traits such as herbicide resistance, new aromas and other natural products into these species. For example, sweet basil and other *Ocimum* and Lamiaceae members are recognized as being rich sources of anthocyanins (Saito and Harbone 1992), which are of interest for their antioxidant proper-

ties and as a source of unique pigments (Phippen and Simon 1998). The formation of unique pigments in purple basil is, however, genetically unstable (Phippen and Simon 2000b), and genetic transformation technology could be a useful tool in stabilizing their biosynthesis. The metabolism of monoterpenes in mint and sage, also members of the Lamiaceae, are considered attractive targets for genetic engineering (Lange and Croteau 1999), and transformation techniques for mint have been reported (Niu et al. 1998; Diemer et al. 1999). Transformation technology would also be a valuable tool when conducting studies on the biosynthesis of essential oils.

This paper reports the development of an *Agrobacterium*-mediated transformation system for basil (*O. basilicum* and *O. citriodorum*) that may be applicable to other basil species, cultivars and chemotypes, thereby allowing the utilization of biotechnology for these important aromatic and medicinal plants.

Materials and methods

Plant materials and explant

Ocimum basilicum, cvs SBA28 and Purple Ruffles, and *O. citriodorum,* cv Sweet Dani, plants were planted in 4-cm-deep cell flats (96 seeds per flat) containing Redi-Earth Coir Mix potting soil and grown in a mist-house under 80% relative humidity and 24°C for approximately 2 weeks, after which they were transferred to a greenhouse [24°C/21°C, 13/11 h (day/night cycle)] for 4 weeks. After 4 weeks in the greenhouse, the first two pairs of leaves (2 cm in length) were collected and used as the explant source for our investigation. Previous regeneration experiments in our laboratory showed that the lower half of a 2-cm basil leaf, divided in two explants of 5 mm each (position 1 at 1–5 mm and position 2 at 6–10 mm from the basal region), yielded the highest frequency of shoots (Phippen and Simon 2000a). Leaf explants of this age and from these two positions were therefore used for *gus* expression experiments.

Agrobacterium tumefaciens strains and GUS expression

Agrobacterium strains GV3101 and EHA105, both containing the pBISN1 plasmid, were used. Plasmid pBISN1 has the *gusA*-intron gene under regulation of a chimeric promoter containing a trimer of octopine-activating sequences and promoter regions from octopine and mannopine synthase genes. The plasmid also contains the *nos-nptII* gene conferring Kan resistance to transgenic plants (Narasimhulu et al. 1996). A*grobacterium* colonies were grown initially at 28°C on YEP solid medium (Chilton et al. 1974), pH 7.0, containing 50 mg l^{-1} Kan and 10 mg l^{-1} rifampicin. A 5-ml culture of a single colony was grown overnight in YEP liquid medium containing the same antibiotics at the same concentrations. A 0.5-ml aliquot of this culture was transferred into 50 ml of AB medium (Chilton et al. 1974), and the growth was monitored until an absorbance (600 nm) of 0.8–1.0 was achieved.

Transformation and selection

Leaves were surface sterilized in 1% (w/v) sodium hypochlorite solution [20% (v/v) Clorox] for 5 min and rinsed three times with sterile water. After sterilization, two small explants (5×5 mm) prepared from the leaf base were used for transformation. A total of 2,200 explants of cv SBA28, 1,900 of cv Purple Ruffles and 2,440 of cv Sweet Dani lemon basil were inoculated by immersion for 30 min in the *Agrobacterium* solution in petri dishes.. The regeneration protocol described by Phippen and Simon (2000a), with slight modifications, was used. The explants were removed and blotted dry with sterilized filter paper and transferred (20 explants per petri dish) to callus and shoot induction medium containing MS (Murashige and Skoog 1962) basal salts and vitamins plus sucrose [3% (w/v)], supplemented with TDZ (16.8 μ*M*). The explants were co-cultivated for 3–4 days, at which time they were transferred to a similar MS liquid medium supplemented with timentin (200 mg l^{-1}) and Kan (30 mg l^{-1}). After 2 weeks, emerging shoots were excised and transferred to a MS medium supplemented with NAA (0.054 μ*M*) instead of TDZ for shoot elongation and root development. An additional period of 3–4 weeks was required for the development of shoots with sufficient root systems to be planted and acclimated to greenhouse conditions.

GUS histochemical assay

The two *Agrobacterium* strains were compared for transformation efficiency by the GUS transient expression assay using leaf explants of sweet basil cv SBA28. The colorimetric GUS histochemical assay (Jefferson and Wilson 1991) was used, and the infection average was calculated from 250 explants in each treatment (five replications of 50 explants; ten explants/petri dish). Leaf explants excised from cultures after 3 days (using the GV3101 strain) or 4 days (using the EHA 105 strain) of co-cultivation with *Agrobacterium* were collected in a solution of 0.1 *M* phosphate buffer (pH 7.5) with 0.1% Triton X-100. For histochemical staining, 1 m*M* X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucoronide) was used as a substrate during an overnight incubation period in darkness at 37°C. Plant material was immersed in 95% ethanol after the incubation period to remove chlorophyll. Endogenous GUS activity was checked visually for blue staining and compared with control basil leaf explants at pH 7.5. For each experiment, tobacco leaf explants were used as a positive control.

Genomic DNA extraction

DNA was extracted from leaves (5 g) using the CTAB procedure (Doyle and Doyle 1990). RNA was removed from the extracted DNA by incubation with RNAase (10 mg ml^{-1}) at 37°C for 1 h.

PCR and Southern blot analyses

To verify stable transformation, genomic DNA was extracted from leaves of putative transgenic plants and analyzed by PCR and Southern blot. Approximately 500 ng of genomic DNA was used as template for the PCR analysis. We used primers 5′-TTATCTCTATGAACTGTGCGTCA-3′ and 5′-TTGGACATACCATCCGTAATAA-3′ to amplify the 697-bp *gus* gene fragment and the primers 5′-TTGAACAAGATGGATTGCACG-3′ and 5′-GATGACAGGAGATCCTGCCC-3′ for the 320-bp *nptII* gene fragment. PCR reactions were performed in 25 μl (total volume) consisting of 1× reaction buffer, 5 m*M* $MgCl_2$, 0.2 m*M* dNTPs, 0.2 μ*M* of each primer and 2 U *Taq* DNA polymerase (Promega, Madison, Wis.). The PCR amplification parameters included an initial 1-min denaturation at 94°C, followed by 30 cycles of 45 s at 94°C, 1 min at 57°C for the *gus* gene and at 56°C for the *nptII* gene, 2 min at 72°C and a final 10-min extension at 72°C, using a PTC-100 programmable thermal controller (MJ Research, Waltham, Mass.). PCR products were separated on 1.0% agarose gels and visualized by staining with 0.5 μg ml^{-1} ethidium bromide. For Southern blot analysis, 20 μg of genomic DNA and 1 μg of plasmid were digested overnight with *Bam*HI restriction enzyme. The digested DNA was fractionated on a 0.7% agarose gel, denatured (1 *M* NaCl plus 0.5 *M* NaOH), neutralized (1.5 *M* NaCl, 0.5 *M* Tris-HCl, pH 7.0) and transferred to nitrocellulose (Sambrook et al. 1989). It was then transferred to a nitrocellulose filter and cross-linked using a UV oven. The blot was hybridized with a [^{32}P]-CTP-labeled DNA probe produced by PCR amplification of the original plasmid using the primers described above for *gus*. Hy-

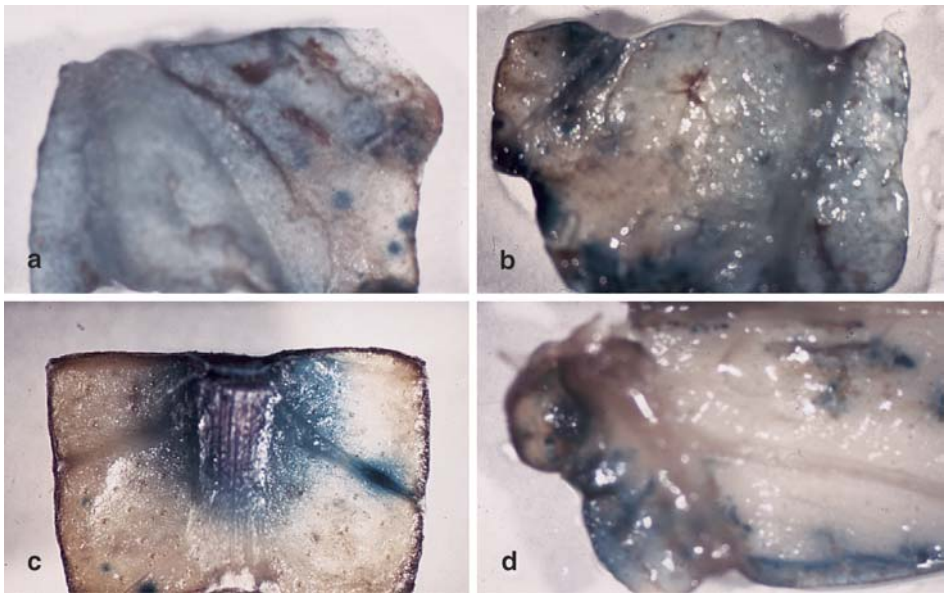

Fig. 1a–d Transient expression of *gus* in *Ocimum basilicum* (cv SBA28) leaf tissue after 3 days of infection with *Agrobacterium tumefaciens*, strain GV3101 (**a**, **b**) and EHA105 (**c**, **d**)

bridization was carried out at 42°C overnight in 50% formamide, 6× SSPE, 5× Denhardt's solution and 0.5% SDS. The membrane was then washed three times at 42°C for 10 min in the following solutions: 2× SCC (saline-sodium citrate, 3 *M* NaCl, 0.3 *M* Na_3-citrate.$2H_2O$) plus 0.2% SDS, 0.5× SCC plus 0.1% SDS and 1× SCC plus 0.1% SDS. The washed blot was visualized utilizing exposure to Kodak films (Biomax MS, 20.3×25.4 cm) for 12 h.

Inheritance of transgenes

Transgenic plants were grown under greenhouse conditions using the same system as described above. Basil seeds from selfed transgenic and non-transgenic *O. basilicum*, cv SBA28, and *O. citriodorum*, cv. Sweet Dani, were harvested from individual plants. All plants were kept in isolation from each other to avoid cross pollination. T1 plants (first generation of transgenic plants) and S1 plants (first generation of non-transgenic plants) of each cultivar were sown and the seedlings grown under the same conditions as for the explant donor stocks.

Extraction of essential oils and gas chromatography/mass spectrometry (GC/MS) analysis

After the transgenic status of the plants with respect to GUS integration had been confirmed using the GUS assay, the essential oils were extracted from the leaves of plants at the identical developmental stage of six pairs of leaves. Three samples of dried tissue (200 mg) were collected from at least ten individual T1 transgenic plants and from ten non-transgenic plants. Samples from transgenic and non-transgenic plants were kept separated for a comparative analysis. Extraction was carried out overnight at 25°C in 5 ml MTBE (*tert*-butyl methyl ether) containing 0.1 µl ml^{-1} of safrole as an internal standard, in 25-ml vessels, with shaking at 30 rpm (Boekel-Fisher Ocelot rotator). The extract was cleaned by passage through a small (Pasteur pipette) column containing anhydrous Na_2SO_4 and silicic acid (Silicagel 60, 230–400 mesh, Merck Darmstadt, Germany). The sample was further cleaned by being passed through a 0.2-µm Millipore filter. The volatile oils were analyzed by gas chromatography coupled to mass and FID detectors (Agilent GC System 6890 series, Mass Selective Detector, Agilent 5973 Network, FID detector). Samples were injected with an autosampler (Agilent 7683 series) utilizing an HP5-MS (30 m, 0.25 ID, 0.25 mm) column. The inlet temperature was 180°C, while the column programmed temperature was 60°C/1 min, 4°C/min and 200°C/15 min. The helium flow rate was 1 ml min^{-1}. Individual compound identifications were made by matching spectra with those from a mass spectral library (Wiley 275.L), and the identity of each compound was confirmed by its Kovats index (Jennings and Shibamoto 1980) and from literature (Adams 1995).

Table 1 Effect of *Agrobacterium* strains (EHA105 and GV3101) and explant position on the number of explants with transient *gus* expression in sweet basil leaf tissue. The numbers represent the average of five replications of 50 explants each

Agrobacterium strain	Position 1[a] (1–5 mm from basal region)	Position 2 (6–10 mm)
EHA105	48.8a[b]	48a
GV3101	50a	48.6a

[a] Leaf sections were the same size but sampled from two positions
[b] Means with same letter are not significantly different according to Tukey's test at 5%

Statistical analysis

Analysis of variance for transient *gus* expression and essential oil concentration as well as the Tukey's test ($P<0.05$) of mean comparison procedures were performed using JMP software, release 3.2.6.

Results and discussion

With a 96% or higher infection rate, plasmid pBISN1 in either *Agrobacterium* strain GV3101 or strain EHA105 was effective in inducing basil *gus* transient expression (Table 1). Given the high rate of infection efficiency, this lack of difference in infection efficiency between the original positions of the explants confirms that both explant positions can be efficiently used for basil transformation.

Although we found no differences in the number of explants showing *gus* expression between either vector,

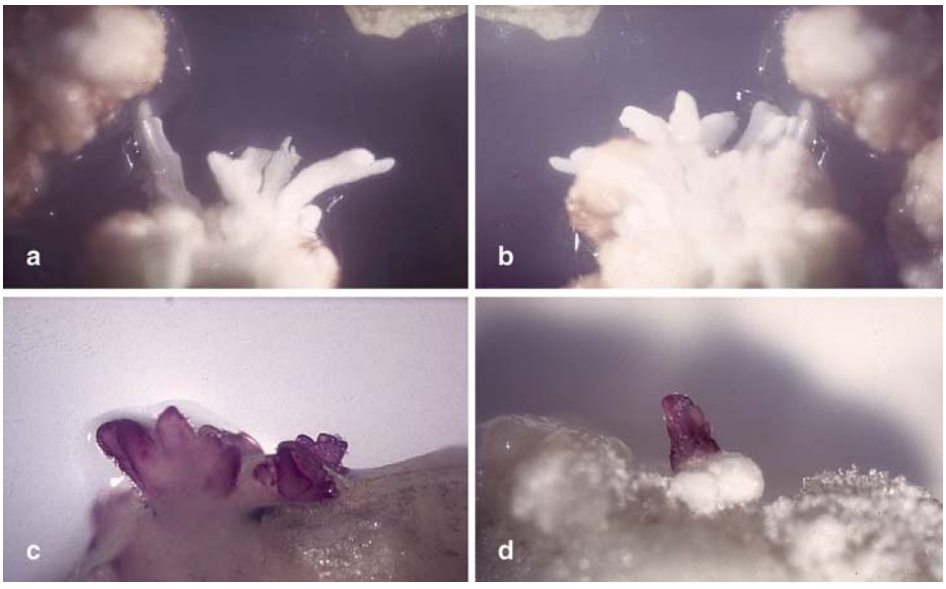

Fig. 2a–d Developing shoots of *O. basilicum* cv SBA28 (**a**, **b**), Purple Ruffles (**c**) and *O. citriodorum*, cv Sweet Dani (**d**), 2 weeks after infection with *A. tumefaciens*

Table 2 Essential oil concentration in leaves of *Ocimum basilicum* (cvs SBA28 and Purple Ruffles) and *O. citriodorum* (cv Sweet Dani) T1 and S1 plants

	Essential oil concentration (μg g^{-1} DWt)				
	1,8-Cineole	Linalool	Eugenol	Neral	Geraniol
O. basilicum					
Transgenic	20.86a[a]	118.98a	69.30a	–	–
Non-transgenic	17.16a	122.27a	75.27a	–	–
O. citriodorum					
Transgenic	–	–	–	468.61a	649.10a
Non-transgenic	–	–	–	488.76a	664.51a

[a] Means with same letter within columns are not significantly different according to Tukey's test at 5%

we found that explants co-cultivated with GV3101 showed a greater intensity and wider distribution of blue areas on the explants. The staining of explants transformed with EHA105 was limited to the region of the main vascular tissue and borders of the explant, while GV3101 gave rise to more generalized staining of the tissue (Fig. 1). The efficiency of the pBISN1 promoter in transient expression experiments with peppermint (*Mentha* × *piperita*) leaf tissue was reported by Niu et al. (1998). Co-cultivation of peppermint leaf explants with EHA 105 resulted in a greater transient expression of *gus* in leaf explants when the reporter gene had the pBISN1 promoter instead of the CaMV 35S promoter.

The T-DNA transfer process is initiated when *Agrobacterium* perceives certain phenolic and sugar compounds from wounded plant cells (Hooykaas and Beijersbergen 1994) that act as inducers of the bacterial *vir* genes (Gelvin 2000). The more complete infection by GV3101 may be in part related to a higher sensitivity to the exudation of specific compounds from basil leaf tissue. Differences in the efficiency of infection in transformation experiments with other members of Lamiaceae family, *Mentha spicata* and *M. arvensis*, was observed by Diemer et al. (1999), who reported that in both species, strain EHA105 was more efficient than the GV2260 strain. Basil explants co-cultivated with *Agrobacterium* started producing callus 7–10 days after infection on solid MS medium containing Kan (30 mg l^{-1}). The percentage of explants producing *Kan*^{r} callus was 92%, 90% and 83% for sweet basil cvs SBA28 and Purple Ruffles and lemon basil cv Sweet Dani, respectively. Control experiments with non-transformed basil leaf tissue had no callus formation on medium containing kanamycin. Normal shoots developed from the transformed callus, and one line was regenerated for each cultivar of *O. basilicum* and for the two lines of *O. citriodorum* on selection medium containing Kan (Fig. 2). In addition, some abnormal shoots were observed but these could not be regenerated.

After 2 months of in vitro cultivation, the plants (5–10 cm in height) were removed from MS medium containing NAA and Kan. The root system was washed with distilled water and the plants planted in Redi-Earth Coir Mix. Plants were initially placed in the mist house for 1 week and then transferred to a greenhouse. Leaves from the four genetic lines were collected for the GUS histochemical assay and molecular analyses. GUS expression was positive for all of the leaves analyzed, occurring over the entire leaf (Fig. 3). High and stable GUS activity was also obtained by Niu et al. (1998) in peppermint leaf tissue transformed with this vector containing the pBISN1 promoter.

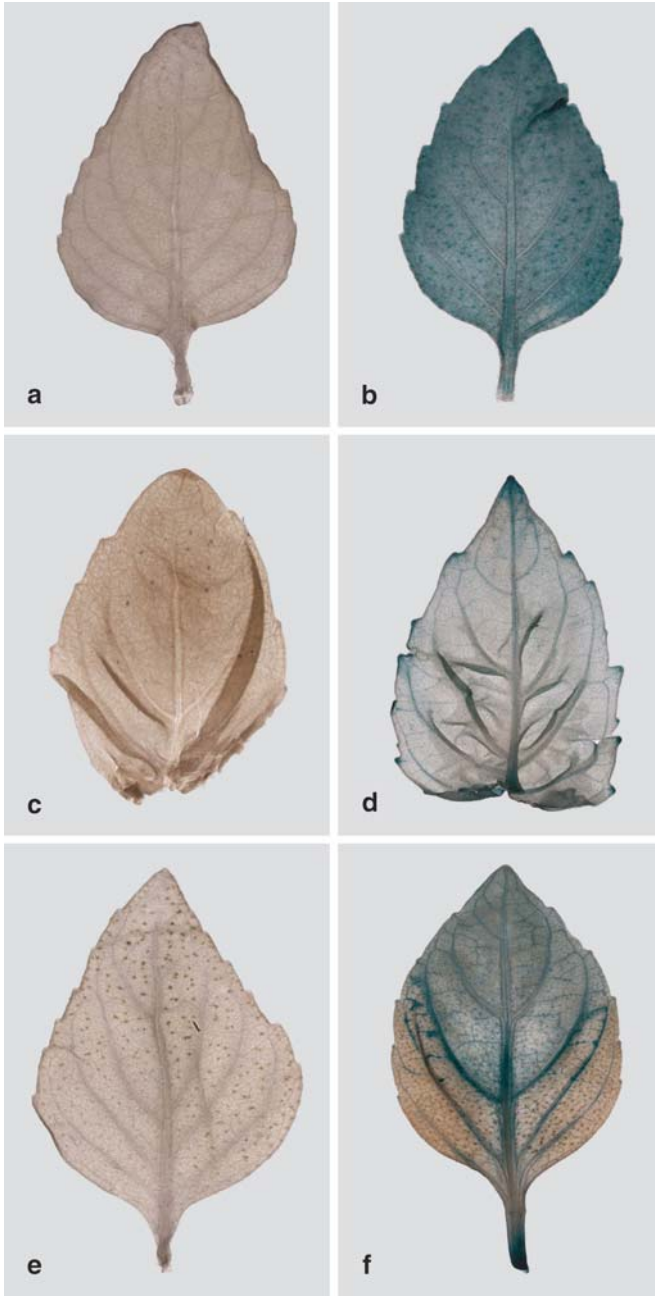

Fig. 3a–f Leaves of sweet basil adult plants exhibiting GUS activity: *O. basilicum*, cvs SBA28 (**a** non-transgenic, **b** transgenic) and Purple Ruffles (**c** non-transgenic, **d** transgenic), and *O. citriodorum*, cv Sweet Dani (**e** non-transgenic, **f** transgenic)

The expected amplified fragment was obtained for *gus* (697 bp) and *nptII* (320 bp) in all of the transformed and regenerated plants (Fig. 4). Proof of integration of the *gus* gene into the basil cultivar chromosomes was obtained by Southern blot after single digestion with *Bam*HI (Fig. 5). From 41 plants transferred to the greenhouse, 37 transgenic basil plants survived and were phenotypically normal (Fig. 6).

Similar Mendelian patterns of segregation were observed in both of the sweet basil cultivars, SBA28 and

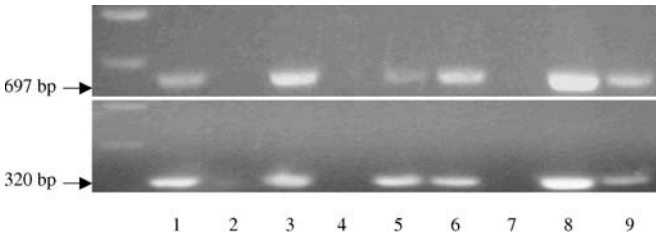

Fig. 4 PCR amplification of 697-bp *gus* and 320-bp *nptII* gene fragment of T0 sweet basil plants. *Lanes 1*, *2 O. basilicum*, cv SBA28, transgenic (*lane 1*) and non-transgenic (*lane 2*); *lanes 3, 4* cv Purple Ruffles, transgenic (*lane 3*) and non-transgenic (*lane 4*); *lanes 5–7 O. citriodorum*, transgenic (*lanes 5, 6*) and non-transgenic (*lane 7*); *lanes 8, 9* positive controls, pBISN1 plasmid (*lane 8*) and *gus* and *nptII* transgenic tobacco (*lane 9*)

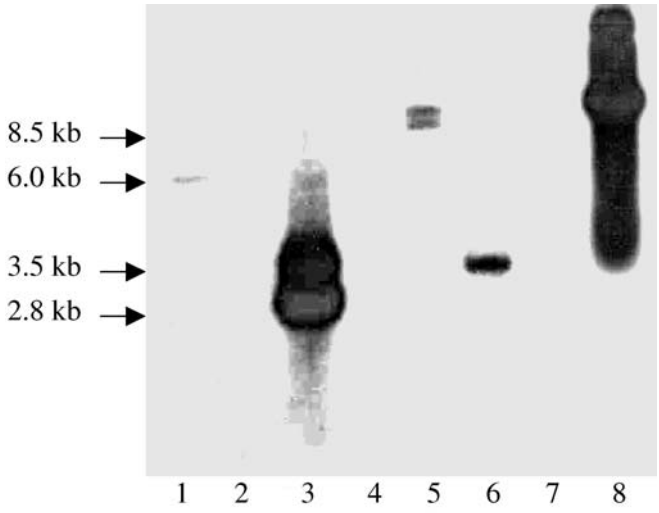

Fig. 5 Southern blot analysis of sweet basil plants genomic DNA digested with *Bam*HI and hybridized to the *gus* probe. *Lanes 1,2 O. basilicum*, cv. SBA28, transgenic (*lane 1*) and non-transgenic (*lane 2*); *lanes 3, 4* cv Purple Ruffles, transgenic (*lane 3*) and non-transgenic (*lane 4*); *lanes 5–7 O. citriodorum*, transgenic (*lanes 5, 6*) and non-transgenic (*lane 7*); *lane 8* pBISN1 plasmid

Fig. 6 Transgenic Sweet Basil SBA28 (*left*), Purple Ruffles (*center*) and Sweet Dani (*right*) plants successfully grown in the greenhouse

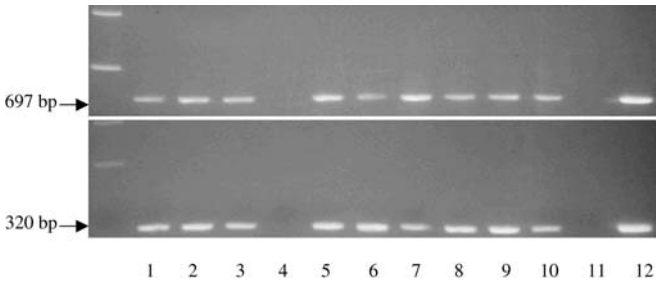

Fig. 7 PCR amplification of 697-bp *gus* and 320-bp *nptII* gene fragment of T1 sweet basil plants. *Lanes 1–4 O. basilicum*, cv SBA28, transgenic (*lanes 1–3*) and non-transgenic (*lane 4*); *lanes 5–11 O. citriodorum*, transgenic line 1 (*lanes 5–7*), transgenic line 2 (*lanes 8–10*) and non-transgenic (*lane 11*); *lane 12* positive control, pBISN1 plasmid

Sweet Dani. Out of the 32 T1 plants analyzed for GUS activity, 23 showed the expected 3:1 ratio ($P<0.05$). PCR analysis of the three randomly selected T1 plants with positive GUS expression for each of cv SBA28 and cv. Sweet Dani (lines 1 and 2) showed amplification of the *gus* and *nptII* gene fragments as expected (Fig. 7).

No phenotypic nor essential oil compositional differences were observed between the transgenic and non-transgenic plants of either basil cultivar. With regard to the volatile essential oils, the T1 transgenic plants accumulated the same essential oil pattern as non-transformed S1 plants (Table 2). In sweet basil cv SBA28, the major volatile oil constituents were 1,8-cineole, linalool and eugenol; while in lemon basil cv Sweet Dani, the major constituents, as expected, included neral and geraniol (citral).

This paper reports the development of an *Agrobacterium* transformation system for basil that shows stable inheritance of the transgene. Though we have developed a very efficient technique for transient expression, further work towards optimizing the regeneration protocol for each cultivar and species is needed. Transgenic plants were obtained using different basil chemotypes and species, and this system will now allow the use of biotechnology in this genus.

Acknowledgements This research was supported by the funding from the Center for New Use Agriculture and Natural Products Program, the New Jersey Agricultural Experiment Station, Cook College, Rutgers University, and the Department of Horticulture and Landscape Architecture, Purdue University. The authors acknowledge the funding by CAPES – Brazilian Agency Foundation for Research, Brazil – who sponsored the senior author's graduate studies in the United States. We also gratefully acknowledge Dr. W. Phippen for his prior work and assistance, Dr. S. Gelvin for his advice and donation of *Agrobacterium* strain GV3101 and pBISN1 plasmid, and Dr. M. Hasegawa for providing the *Agrobacterium* strain EHA105 for these studies. We thank Dr. N. Dudareva, Dr. F. Ballenger, Dr. Y. Tadmor and J. Kapteyn for their comments on this research and manuscript.

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